VARIATION AND DIVERSIFICATION IN THE SEXUAL SIGNALS OF SPADEFOOT TOADS

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ABSTRACT

Rebecca O'Brien: Variation and Diversification in the Sexual Signals of Spadefoot Toads
(Under the direction of Karin Pfennig)

Explaining the origins of diversity is an enduring goal in evolutionary ecology, and in few places is this diversity as striking as in sexual signals. One significant pressure driving signal design is the presence of competitors. These competitors may be either of the same species and competing for resources such as mates, or of different species and competing for other resources such as signal space. In my first chapter, I explore how changes in signal design, driven by reproductive character displacement, can be facilitated by changes in habitat. In my second, I explore the role of signal design in determining facultative signal adjustment in response to interference from competitors. I find that differences in ecoregion can cause variation in reproductive character displacement throughout a range of sympatry, and that signal structure plays an important role in determining how animals facultatively respond to signal interference.

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INTRODUCTION

Sexual signals are shaped by a complex network of selective pressures acting at the level of signal production, signal transmission, and receiver response (Endler, 1992). The various combinations of these pressures have resulted in a stunning diversity of signals that span nearly every sensory modality, and range from simple changes in body size, to complex combinations of stimuli appealing to multiple sensory systems.

This diversity in signals plays an important role species richness. Sexual signals have the potential not only to maintain species boundaries by reducing hybridization (Dobzhansky, 1940), but also to actively increase diversity by contributing to speciation (Kraaijeveld et al., 2011; West-Eberhard, 1983; Coyne and Orr, 2004).

Still, despite the importance of sexual signals in evolutionary processes, there is much that remains to be learned about what drives signal diversity. In particular, untangling the knot of selective pressures that shape signal design has proved to be no easy task (Wilkins, 2013), and there is much that remains unclear about the processes of signal divergence (Ritchie, 2007). In this thesis, I explore the factors contributing to signal diversity using the plains spadefoot toad, *Spea bombifrons,* as a model system. In particular, I focus on the role of interactions between competitors of both the same and of different species in driving signal divergence.

In my first chapter, I explore how changes in signal design, driven by reproductive character displacement, can be facilitated by changes in habitat. In my second, I explore facultative signal adjustment in response to signal interference by competitors. I find that differences in ecoregion can cause variation in reproductive character displacement throughout a

range of sympatry, and that signal structure plays an important role in determining how animals facultatively respond to signal interference.

Study System

The plains spadefoot toad *Spea bombifrons*, is a small anuran with an extensive range that stretches from Canada south through the mid-west and into the desert southwest of the United States (Powell, 2016). Spadefoots are explosive breeders that spend most of their lives underground and emerge following heavy rains to breed. Males call to attract females, and upon choosing a mate, females initiate amplexus by touching their chosen male (Bragg, 1965).

In the southern portion of its range, *S. bombifrons* comes into contact with another member of the *Spea* genus, *Spea multiplicata,* with which it is capable of hybridizing (Forester, 1975; Pfennig and Simovich, 2002). While the offspring of these pairings exhibit reduced fitness (males can be sterile and females are only partially fecund; Simovich et al., 1991) hybrids also develop more quickly than pure *S. bombifrons* (Pfennig and Simovich, 2002). In the desert southwest, where ponds often dry before tadpoles have time to metamorphose (Bragg, 1965; Pfennig, 1992; Pfennig and Simovich, 2002), this rapid development is advantageous, and can outweigh the cost of reduced fecundity (Pfennig, 2007). Although females of *S. multiplicata* consistently prefer their own species, female *S. bombifrons* exhibit facultative mate choice where they are equally likely to mate with *S. multiplicata* or *S. bombifrons* in low-water conditions (Pfennig, 2007).

Previous research has found evidence of reproductive character displacement of female choice in *S. multiplicata* and of male aggregation behavior in *S. bombifrons* (Pfennig and Rice, 2014; Pfennig and Steward, 2011). *S. bombifrons* males also show remarkable divergence in call

characteristics between allopatry and portions of sympatry (Pierce, 1976) which has frequently been attributed to character displacement (e.g. Pfennig and Steward, 2011; Pfennig and Pfennig, 2005). While they have slow, snoring calls in much of their range, in southern New Mexico and Arizona, their calls are significantly faster and have a quacking quality. The causes of this divergence are further explored in this thesis.

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CHAPTER 1: VARIABLE MATING SIGNAL DIVERGENCE ACROSS SYMPATRY IN THE PLAINS SPADEFOOT TOAD, *SPEA BOMBIFRONS*

Introduction

Reproductive character displacement (RCD) is the process of signal divergence in regions of sympatry due to the presence of a heterospecific (Brown and Wilson, 1956; Grant, 1972; Howard, 1993; Pfennig and Pfennig, 2012). This divergence can be driven by signal interference, where overlapping signals obscure one another and hinder mate localization (Birch, 1957; Butlin and Ritchie, 1994; Gerhardt and Huber, 2002), or by signal confusion, where the inability to distinguish between signals leads to deleterious hybridization—a process known as reinforcement (Dobzhansky, 1937, 1940; Blair, 1974).

The magnitude of character divergence can vary both between (Cooley, 2007) and within (Pfennig and Pfennig, 2012) species, but previous studies have been largely concerned with differences between species. This has been primarily driven by a prolonged interest asymmetric character displacement, where only one of the interacting species exhibits character divergence (*Reviewed in* Cooley, 2007). However, intraspecific variation within a range of sympatry is also common and deserving of further attention.

Improved understanding of when and how variation in the presence of RCD within sympatry can arise is important for at least two reasons: First, varying levels of RCD may result in misestimation of the frequency with which reinforcement occurs. Most studies testing for reinforcement have taken the approach of sampling the sexual signals of individuals from select

populations in sympatric and allopatric environments and looking for patterns of divergence between the two regions (Lack, 1947). If levels of displacement vary within a range of sympatry, minimal sampling may fail to detect evidence of character displacement that is indeed present (Simberloff and Boecklen,1981; Gabor and Ryan, 2001). Second, variable levels of character displacement may complicate the role of RCD in driving speciation. If different regions of sympatry have varying levels of signal divergence, this means that RCD may drive reproductive isolation not only between sympatric and allopatric populations (Howard,1993), but also between different groups within a region of sympatry

There are several potential causes of variation within sympatry that have been put forward. These include variation in the risk of hybridization (due to factors such as relative species abundance; Howard 1993; Pfennig and Pfennig, 2012; Noor 1995; Peterson et al., 2005); patterns of gene flow (Servedio and Kirkpatrick, 1997; Case and Taper, 2000; Nosil et al., 2003; Goldberg and Lande, 2007); and variation in hybrid fitness (Liou and Price, 1994). Importantly, many of these causes of variation in RCD may covary, changing throughout the range of sympatry as the result of biotic and abiotic factors. For example, if the range of sympatry crosses multiple ecosystems, this can influence each of the above factors and may magnify signal divergence in some areas while reducing it in others. By looking for regions of exaggerated divergence, assessing the role of each of these potential causal factors in driving that divergence, and then testing for an overarching effect of habitat, we can better understand how variation in RCD evolves.

Here, we explore the potential for variation in the strength of selection for RCD in driving divergence of the sexual signals of the plains spadefoot toad, *Spea bombifrons*. *S. bombifrons* is a small anuran with an extensive range that stretches from Canada south through

the mid-west and into the desert southwest of the United States (Powell, 1965). As a drought-tolerant species, *S. bombifrons* spend most of their lives underground and emerge following heavy rains to breed (Bragg, 1965). Males call to attract females to breeding aggregations, and upon choosing a mate, females initiate amplexus by touching their chosen male (Bragg, 1965).

There are several things that make *S. bombifrons* an excellent system in which to explore variation in RCD. First, due to a range expansion from its ancestral habitat in Oklahoma (Rice and Pfennig, 2008), *S. bombifrons* comes into contact with a congeneric, *S. multiplicata*, in the southern reaches of its range. The range of sympatry between these two species is extensive, and spans multiple ecoregions, providing an excellent opportunity to assess the role of habitat in driving variation in RCD.

Second, there is evidence of RCD between *S. bombifrons* and *S. multiplicata*. The two species are capable of hybridizing to produce offspring of reduced fecundity (Forester, 1975; Pfennig and Simovich, 2002), and there is previous evidence of RCD in female choice and male aggregation

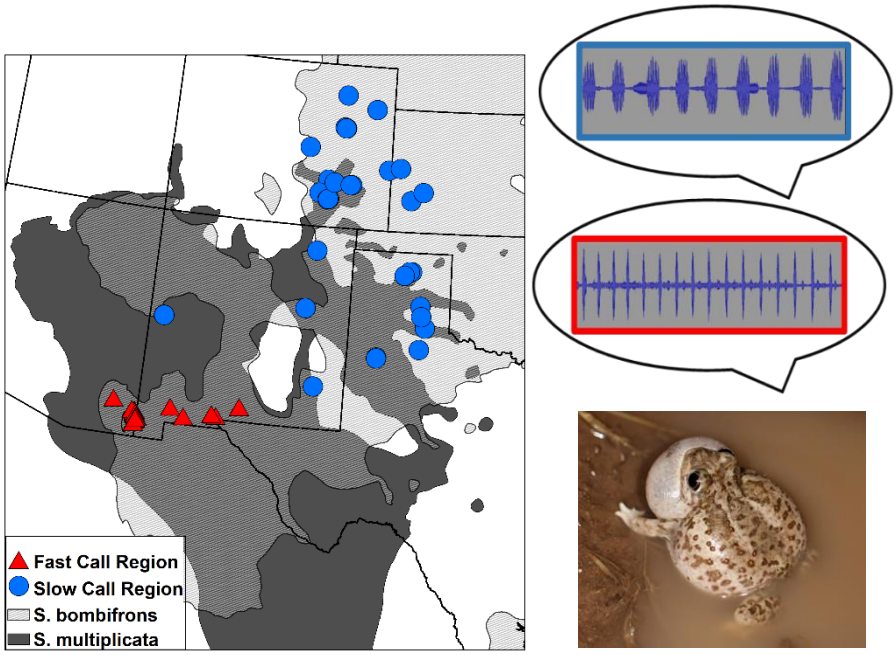

Figure 1.1 *S. bombifrons* has variable calls throughout its range, with slow calls in the northern parts of its range (shown on the map in blue and in the top waveform) and much faster calls in the southern parts of its range (shown in red and in the bottom waveform). The range of S. mutliplicata is shown in dark gray and the range of *S. bombifrons* is shown in light gray.

behavior in the system (Pfennig and Rice, 2014; Pfennig and Steward, 2011). These previous studies provide strong evidence of a selective pressure for RCD between the two species.

Finally, *S. bombifrons* also exhibits a striking pattern of call divergence throughout its range (Pierce, 1976; Figure 1.1). While males have slow, snoring calls throughout the Northern part of their range and into the Midwest, the calls in the desert southwest are much faster, and have a quacking quality, making them distinctly different (Pierce, 1976).

In this study, we investigate variation in the presence of reproductive character displacement throughout sympatry in the plains spadefoot toad by: 1) Describing *S. bombifrons'* call characteristics and calling locations throughout allopatry and sympatry with *S. multiplicata*. 2) Determining the nature of the transition between call types within the range of sympatry (i.e. Is the transition a gradual cline or an abrupt switch?) 3) Testing for potential correlations between ecoregion and variation in RCD. 4) Exploring the interplay between ecoregion and known causes of variation in RCD including the risk of hybridization, variation in hybrid fitness, and patterns of gene flow. We also discuss other potential causes of signal variation in this system.

Methods

Field collections

Between 1996 and 2017 we recorded the calls of 110 *S. multiplicata* and 191 *S. bombifrons* males from 52 different populations. These ponds were located throughout Texas, Colorado, Kansas, New Mexico, and Arizona. We recorded each spadefoot for a minimum of 30 seconds at a sampling rate of 44.1 KHz. After recording, we caught, weighed, and measured each male, and collected a DNA sample in the form of a toe clip.

Genotyping

In order to ensure that the presence of hybrids did not influence our results, we genotyped each toad to determine its species. Following Pfennig et al. (2012), species identity was determined using a suite of 9 nuclear markers and one mitochondrial marker. Because hybridization can alter call characteristics (Blair,1955; Pfennig, 2000; Lemmon, 2009; O'Brien, unpublished data), we included only those individuals who showed no introgression at any of the 10 markers in our analysis.

Describing call characteristics

Call Analysis

We analyzed each call to determine call rate (calls/minute), pulse rate (pulses/second), pulse number (pulses/call), intercall interval (seconds), and call duration (seconds) using Audacity® sound analysis software 2.1.3 (Audacity team, 2018). Dominant frequency (Hz) was measured using Raven Pro 1.5 (Bioacoustic Research Program, 2014) software. We used a fast fourier transformation with a hamming window, a hop size of 82, and a discrete fourier transform of size 2048.

Visualization of Calling Clusters

As an initial assessment of variation in calls across region and species, we first performed a principle component analysis (PCA) on a cross-correlation matrix including call rate, pulse rate, dominant frequency, and pulse number in JMP Pro 13 (SAS Institute Inc., Cary, NC). We then used normal mixture clustering and compared AICc values to determine the most likely

number of clusters in sound space. We chose the model with the lowest AICc value by a minimum of 4 AICc units, and, in the case of a tie, we chose the model with the fewest clusters.

Testing clinal call variation

Our PCA confirmed that there were two distinct call types: one found in Southern New Mexico/Arizona (hereafter the "fast-call region") and one found throughout the rest of each species' range (hereafter the "slow-call region"). To ensure that this was a distinct difference, and not just the result of a gradual cline, we used analysis of variance (employing the 'Anova' function in the 'car' package in R 3.4.1) on linear mixed models to test for latitudinal clines in both *S. multiplicata* and *S. bombifrons'* call parameters. The North-South orientation of *S. bombifrons'* range expansion meant that this served as a good proxy to determine gradual transitions through space that might have been caused by factors such as drift during range expansion. Our models included both latitude and call type (fast vs. slow) as covariates. To control for the effects of temperature on call rate, call duration, and pulse rate (*Reviewed in*: Gerhardt and Huber, 2002) we included the water temperature as a covariate for these models. We also included body size as a covariate in the model of dominant frequency, as there is a well-established correlation between body size and this call parameter (Zweifel, 1968; McClelland et al., 2006) For those call parameters that were significantly affected by the competitive environment (as determined by the total number of calling males or distance to the nearest neighbor), these covariates were also included in the model (See chapter 2). All models also included the pond from which an individual was collected as a random effect.

Testing for RCD throughout the slow call region of sympatry

Although the PCA and our linear mixed models made clear that there were significant differences between the fast-call region and the slow-call region, we were unable to tell from these models if there was RCD in the Northern parts of sympatry. To determine this, we ran a second set of models including all of the above covariates and adding the covariate of whether the toad was recorded in a sympatric or allopatric environment. This enabled us to determine the effect of sympatry/allopatry on call parameters without our results being entirely driven by the highly diverged calls. We did not have any allopatric *S. multiplicata*, so this analysis was done exclusively in *S. bombifrons.*

Evaluating call location within a pond

In addition to altering signal characteristics, animals will also adjust the timing or location of their signaling to enhance female ability to distinguish between species (Pfennig and Pfennig, 2012). In order to address this potential change, we tested for variation in calling locations throughout the range of sympatry. We estimated each male's calling location as a percent across the pond (for example, calling from the pond's perimeter would be 0% across, while calling from the center would be 50% across). We then used a Mann-Whitney U test ("stats" package in R 3.4.1) to compare the average calling location for *S. bombifrons,* first between sympatric and allopatric environments, and then between the fast- and slow-call regions. We had insufficient records of *S. multiplicata* calling locations for comparison, so we limited our analysis to *S. bombifrons*.

Determining ecoregion's effects on sexual signals

In order to determine if the changes in signaling between the fast-call region and the slow-call region were ultimately linked to changes in ecoregion, we used a combination of field-collected data and large-scale databases to determine the role of habitat in the strength of RCD. The large-scale data enabled us to identify correlations between patterns of displacement and underlying patterns in habitat, while the field data enabled us to look more closely at direct relationships between ecoregion and call divergence.

Ecoregion analysis

For our broadest analysis, we used the Nature Conservancy's terrestrial ecoregions map (TNC Conservation Portfolio) at the level of "Major Habitat Type" in ArcGIS® 10.5 (ESRI, 2016). This dataset determines ecoregions based on their climatic regimes, vegetation structure, spatial patterns of biodiversity, and guild structure (Olson et al., 2002), and provided a broad overview of how ecoregion correlated with changes in RCD. We extracted the major habitat type at each of our sample points and then used a Fisher's exact test to look for correlations between the call type region (fast-call region/slow-call region) and ecoregion. To confirm that our results were not due to spatial autocorrelation, we also ran a linear mixed model on PC1 and PC2 with terrestrial ecoregion as a covariate and a random effect that grouped ponds based on a 20km buffer zone.

To ensure that our results were not due to biases in geographic data, we confirmed our results by re-running the above analyses using the more finely detailed USGS national gap landcover analysis map (USGS GAP). We extracted values from this map at the level of class, which demarcates ecoregion boundaries based on basic moisture, temperature, and/or substrate

conditions. For those readings that did not reflect the underlying ecoregion (e.g. areas designated "Developed and other human use") we assigned the closest natural ecoregion designation to that point.

Following these broad analyses, we then looked more specifically at rainfall, which is the aspect of the ecoregion that we thought most likely to contribute to the change in call type and chorus community. To determine average rainfall, we used the PRISM 30-year normal data set at the 800m resolution (PRISM Climate Group, 2004) and extracted average annual rainfall (mm) at each field site using ArcGIS®. We then ran an Anova on a linear mixed model of PC1 and PC2 with average rainfall as a covariate and a random effect of the 20 km buffer zone.

Field assessments of chorus community

The chorus community can have a strong influence on call characteristics by changing the acoustic background against which a signal is produced and potentially heightening difficulties in signal discrimination (Brumm, 2013; Gerhardt and Huber, 2002.; Wollerman and Wiley, 2002; Bee, 2008). Importantly, this community may covary with changes in ecoregion. In order to account for differences in chorus community, and also to determine if changes in this community may be contributing to variation in the RCD throughout sympatry, we used field-collected data to look for correlations between the strength of RCD and chorus community.

At each breeding aggregation, we recorded each of the species heard calling, and calculated the total number species. For older data which did not include chorus community, we listened through the recordings from those ponds and identified the species present by call. Again, to analyze our results, we used an Anova on a linear mixed model with PC1 and PC2 as covariates and the 20km buffer zone as a random effect.

Relative abundance of S. bombifrons and S. multiplicata

The relative abundance of two hybridizing species can also influence the strength of selection for RCD. If a species is relatively rare in one region of sympatry, and more common in another region, then we would expect stronger selection for RCD in the environment where the focal species is rare and at greater risk of hybridizing (Pfennig and Pfennig, 2012). To test for this effect, we estimated the relative abundance of *S. bombifrons* and *S. multiplicata* (recorded as percent *S. bombifrons*) at each pond. Excluding allopatric ponds from our analysis, we then checked for correlation between region (fast vs. slow) and the relative abundance of each species. We also tested for correlations between relative abundance of *S. bombifrons* and each of the measured call parameters (call rate, pulse rate, dominant frequency, pulse number, and duration.) Finally, in an effort to account for possible difference in species distributions at a broader scale, we also compared the number of ponds with only *S. multiplicata* and no *S. bombifrons* between the two regions.

Results

Call displacement

The results of our PCA confirmed the findings of Pierce (1976) in showing a sharp difference in call parameters between fast-call region and the slow-call region (Figure 1.2). The first three components of our PCA analysis explained 98.27% of our variance (Appendix A Table A1) and resulted in four clusters. These clusters corresponded closely to both region (fast-call or slow-call) and species.

S. bombifrons calls in the fast-call region were produced at significantly faster rates (χ^2= 361.929, df=1, p<.001), were significantly shorter in duration (χ^2= 30.996, df=1, p<.001), and had significantly higher pulse rates (χ^2= 2754.039, df=1, p<.001) and lower pulse numbers

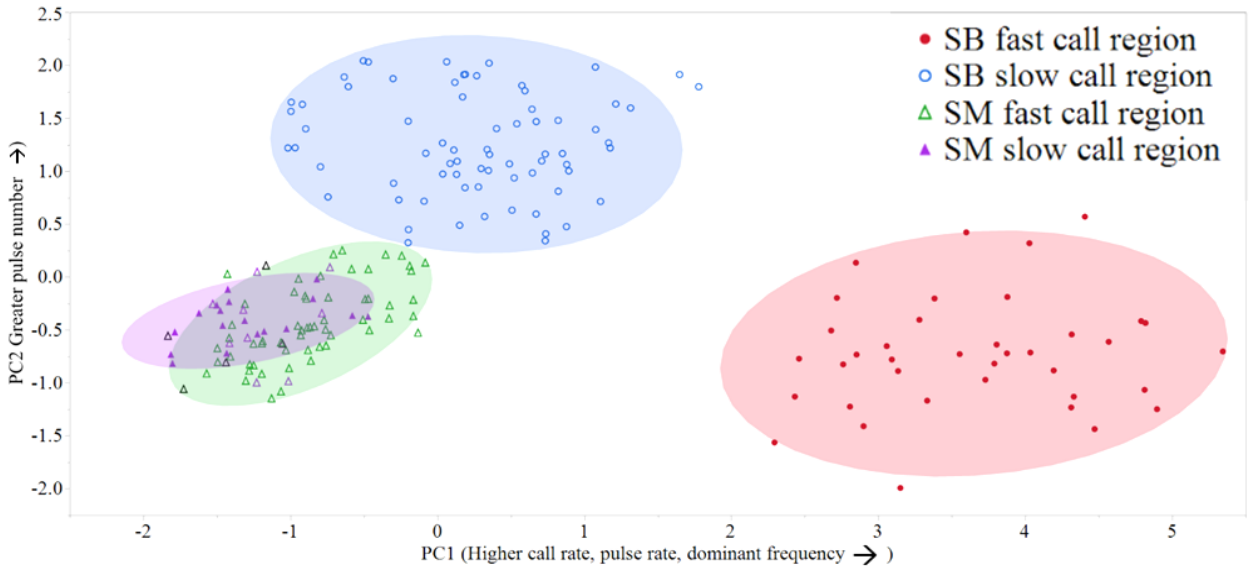

Figure 1.2 A PCA analysis of call parameters for *S. bombifrons* and *S. multiplicata.* Clustering revealed differences between Southern New Mexico/Arizona and the rest of the range in both *S. bombifrons* and *S. multiplicata*. Each symbol represents and individual male. Neither call type nor species was included in the PCA.

(χ^2= 16.558, df=1, p<.001) than calls in the slow call region (Table 1.1). The dominant frequencies in the fast call region were also significantly higher than anywhere else in their range, but this pattern appeared to be the combined result of a latitudinal cline and differences in body size. The pattern did not hold when these factors were included in the model (χ^2=1.185, df=1, p=.276). Intercall interval did not differ between the two regions (χ^2= 2.212, df=1, p=.137).

Table 1.1 Mean and standard error call parameters for fast and slow *S. bombifrons*. Values are not temperature corrected.

	Fast-call region	Slow-call region
Call Rate	38.13 +/- .990	27.68 +/- 0.77
Pulse Rate	60.07 +/- 1.180	44.97 +/- 1.15
Call duration	0.152 +/- 0.003	0.90 +/- 0.03
Pulse number	36.70 +/- .658	36.64 +/- .349
Dominant Frequency	1453.86+/- 21.167	1453.86 +/- 8.88

Our comparison of *S. multiplicata* calls between the fast- and slow-call regions showed that, as in *S. bombifrons*, *S. multiplicata* also had significantly faster call rates in the fast-call region than in the slow-call region (χ^2= 306.301, df=1, p<.001; Figure 1.3).

However, accounting for the effects of distance to the nearest neighbor and total males (see chapter 2), we found that pulse rate (χ^2=0.574, df=1, p= 0.448), pulse number (χ^2=1.260, df=1, p=0.262), and dominant frequency were not significantly different between the two regions (χ^2=.840, df=1, p= 0.359). Intercall interval and call duration (χ^2= 5.278, df=1, p=.073), were also not significantly different between the two regions, though they were nearly so (*Intercall interval:* χ^2= 3.495, df=1, p=0.060 *Call duration*: χ^2= 5.278, df=1, p=.073).

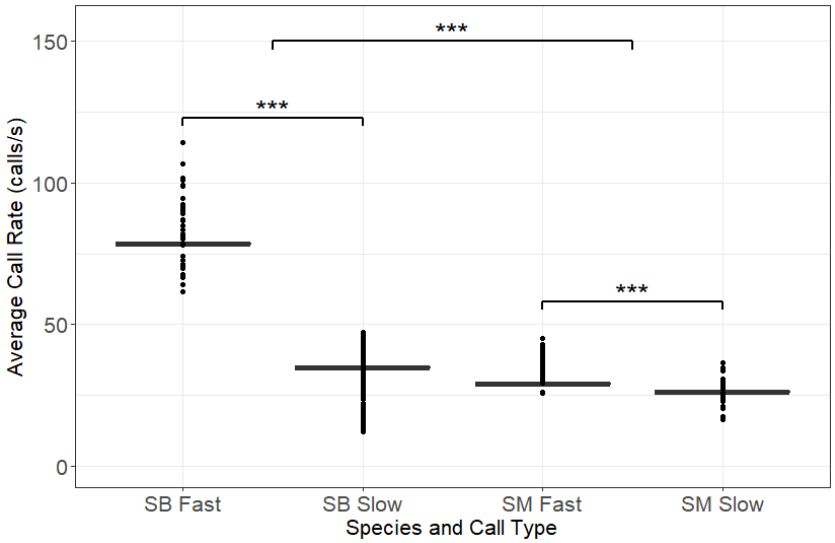

Figure 1.3 Least square means of call rate for fast and slow call *S. bombifrons* (Used only for visualization to minimize effect of water temperature.) All four groups were significantly different from one another. Call rate is one of the most significant factors in female choice (Pfennig 2000).

Both dominant frequency and pulse number showed a latitudinal cline (Appendix A figures A1-5), and this was included in the model for these call parameters. There were no differences in calls between sympatric and allopatric populations of *S. bombifrons* in any of the call parameters (p>.05).

Calling location displacement

We found that *S. bombifrons* are significantly more likely to be calling near the perimeter of the pond in the fast-call region, and near the center of the pond in the slow-call region (W=186, p< 0.05; Figure 1.4). There was not a significant difference in calling location between *S. bombifrons* and *S. multiplicata* throughout the slow call region (W=653.5, p= 0.937), and although we did not have sufficient data to compare *S. multiplicata* calling location between the fast- and slow-call regions, we never observed this species calling from outside of the water on the edge of the pond as we did for *S. bombifrons* (Personal observation and personal communication with K. Pfennig). Accounting for call region (fast vs slow) there was no significant difference in calling location between sympatry and allopatry in *S. bombifrons* (W=880.5, p=0.577).

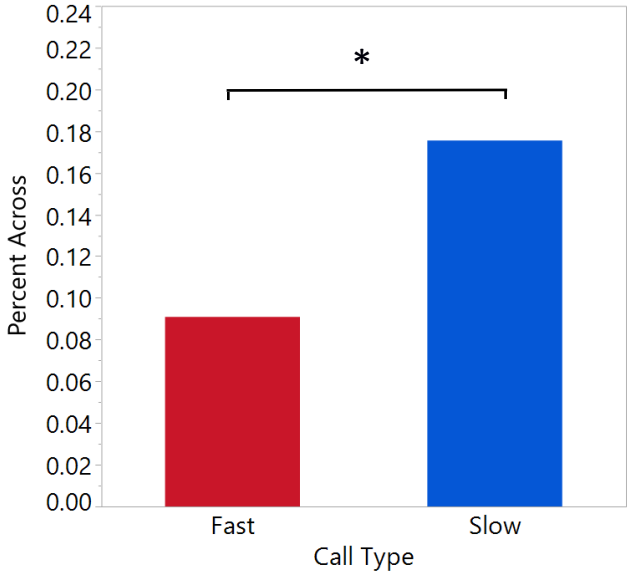

Figure 1.4 There was a significant difference in the average calling locations of *S. bomifrons* between the fast and slow call region. Shown here is the average percent across the pond for each call type.

Environment

Ecoregion was a strong predictor of RCD for both call character and call location using both the The Nature Conservancy ecoregion data (Fisher's exact: p<.001; Anova PC1: χ^2= 16.729, df=3 p<0.001; Anova PC2: χ^2= 28.682 df=3, p<0.001; Figure 1.5) and the USGS landcover data (Fisher's exact: p<.001; Anova PC1: χ^2=8.4205, df=2, p=0.015; Anova PC2:

χ^2=20.298, df=2, p<.001). The fast call region was closely correlated with the desert and xeric shrublands ecoregion, while the slow call region was primarily found in temperate grassland. As expected, this difference in ecoregion translated into differences in rainfall with significantly reduced rainfall in the fast-call region as compared to the slow-call region (χ^2=8.671, df=1, p=0.003).

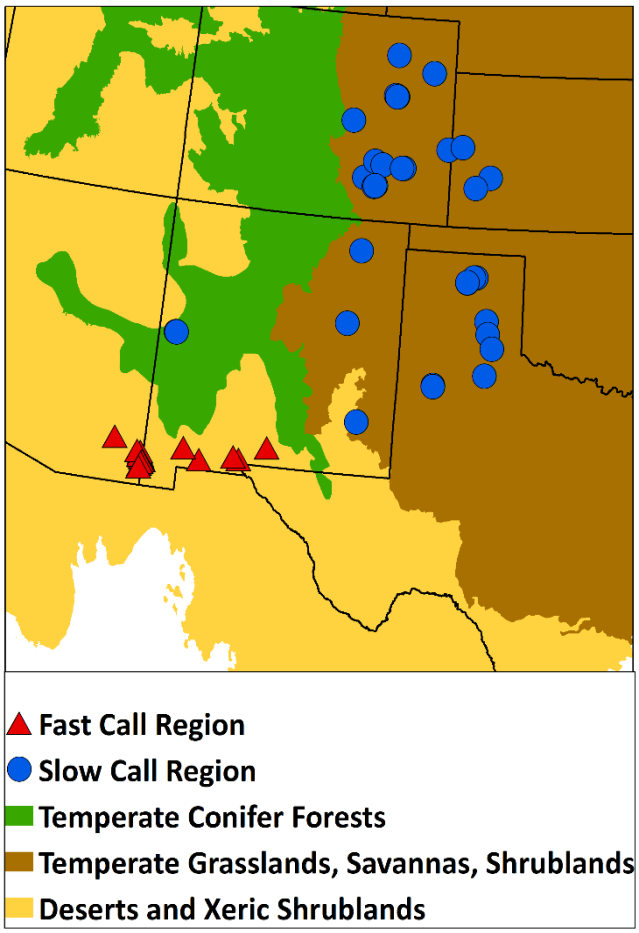

Figure 1.5 *S. bombifrons* fast call type is found exclusively in the desert and xeric shrublands ecoregion, while the slow call type is found primarily in the temperate grasslands, savanna and shrublands ecoregion. Recording locations are shown here overlaying the Nature Conservancy's terrestrial ecoregion map.

Species Present

The fast-call region had significantly higher chorus community richness (t=7.928, df=31.684, p<.001) than the slow call region. This was primarily due to increased prevalence of *Anaxyrus debilis* and *Anaxyrus cognatus*, and *Scaphiopus couchii* in the fast-call ponds as compared to slow-call ponds.

Relative abundance of species

We found no significant difference in the relative abundance of *S. bombifrons* and *S. multiplicata* at the pond level between the fast- and slow-call regions (t=-0.539, df=28.004, p=.594). However, we did find a nearly significant difference in the number of entirely *S.*

multiplicata ponds between the fast- and slow-call regions, with more pure-*S. multiplicata* ponds in the fast-call region than the slow-call region ($t = 2.064$, $df = 17.775$, $p = 0.054$).

Looking at each call parameter independently, we only found a significant effect of percent *S. bombifrons* on pulse number in *S. multiplicata* (χ^2=9.464, df=1, p=.002) with decreasing pulse number corresponding to increasing percent *S. bombifrons*. None of the other call parameters in either species were affected (p>.05).

Discussion

S. bombifrons shows a striking divergence in call type across its range of sympatry with *S. multiplicata.* While *S. bombifrons* has slow calls in much of its range, the calls in the southern-most portion are significantly faster. There are several pieces of evidence to suggest that this divergence is due to reproductive character displacement. First, there is known hybridization between *S. bombifrons* and *S. multiplicata* (Forester, 1975; Pfennig and Simovich, 2002), and this hybridization is known to have negative effects on offspring fecundity (Simovich et al., 1991). Second, there is evidence of RCD in other aspects of this system (Pfennig and Steward, 2011; Pfennig and Rice, 2014), confirming that the selective pressure exists. Finally, these changes increase the difference between the sexual signals of *S. bombifrons* and those of *S. multiplicata*—a pattern consistent with RCD.

The variable strength of RCD found throughout sympatry in this system may have several different causes, many of which are closely tied to the differences in habitat between the slow-call region into the fast-call region. While there is little to no pattern of RCD in *S. bombifrons* throughout the northern stretch of sympatry, which is primarily temperate grassland, there is a strong pattern of divergence in both calling location and call character in the southern-most part of sympatry where it crosses into desert habitat. In this arid region, *S. bombifrons* call

significantly closer to the edge of the pond than they do in the rest of their range, and the calls are significantly faster.

One driver of RCD strength that is likely influenced by the change in habitat is the risk of hybridization. Increased risk of hybridization should result in greater pressure for signal divergence (Pfennig and Pfennig, 2012.) If risk varies throughout a range of sympatry, this can vary the strength of selection for RCD. Hybridization risk can be influenced by the species ratios (with more skewed ratios increasing the risk of hybridization for the less common species) or the amount of contact between the two species, a factor that can be independent of relative abundance. Although we did not find any difference in the species ratios between the two areas, we did find evidence of differences in contact, with increased contact in the arid environments. *Spea* depend on the ponds that form following heavy rain for breeding habitat (Bragg, 1965), and the less rainfall an area receives, the smaller the number of suitable breeding sites is likely to be. This means that the arid environments, where we see significant call divergence, are also the places where the two species interact most and are most at risk for hybridization (Pfennig and Simovich, 2002; Marquez-Garcia et al., 2009).

Differences in habitat may also contribute to differences in the strength of RCD by influencing gene flow. Gene flow from allopatry can impede the evolution of signal divergence in sympatry by washing out adaptive alleles. If the amount of gene flow varies across a region, this can cause differences in the amount of signal divergence that occurs.

As amphibians, *S. bombifrons* are highly dependent on rainfall to make above-ground environments habitable and enable migration (Bragg, 1965). The less rain an area receives, the fewer wet periods there will be when *S. bombifrons* is able to cover large distances, and the less gene flow there is likely to be between populations. The differences in signal divergence

between the slow call region and the fast call region may be the result of allopatric gene flow prohibiting divergence in northern sympatry where there is more rainfall and it is easier for toads to move between populations and permitting gene flow in southern sympatry where the dry environments limit toad mobility. This possibility is supported by previous research which has shown a pattern of greater population structure and reduced gene flow in the fast-call region as compared to the rest of the species' range (Pierce et al., 2017).

Differences in habitat also mean differences in species assembly, and this may also contribute to the strength of selection for RCD by influencing the signaling environment. There are far more anuran species found in the fast-call region ponds than the slow-call region ponds, and among the species found at higher densities in the fast-call region are *Anaxyrus debilis* and *Anaxyrus cognatus.* Both of these species have extremely loud calls (A field recording of a calling *A. cognatus* produced a volume of 109 db at a 1ft distance—loud enough to cause hearing damage after more than 30 minutes of exposure; OSHA, 1970), and when multiple males of these species are calling at once, the ponds become deafening. This increased background noise may impede the ability of female toads to discriminate between species and increase the risk of hybridization (Wollerman and Wiley, 2000; Bee, 2008.) This difficulty is likely compounded by the fact that *S. multiplicata* in this region have slightly faster calls, make them more similar to the calls of *S. bombifrons.*

Habitat can also influence hybrid fitness which can, in turn, influence the strength of selection for RCD (Liou and Price,1994). If hybrids do better in one environment than the other, that can influence the strength of selection for RCD in the different environments. This means that we would expect the hybrids in the fast-call region, where calls are displaced, to have lower fitness than in the slow-call region. However, this does not seem to be the case. Hybrid tadpoles

develop more quickly than pure *S. bombifrons* tadpoles (Pfennig and Simovich, 2002), meaning that in very arid environments where ponds often dry before tadpoles escape, it can actually be beneficial to be a hybrid (Pfennig, 2007).

Still, we do not feel that this discounts our hypothesis, as there are two explanations for why we may still see greater RCD in this region: First, and most importantly, there is evidence that, unlike hybrid males in the fast-call region, hybrid males in the slow-call region are not entirely sterile (Forester, 1969, 1975). This suggests that there may be more relaxed selection against hybridization in this region, reducing the need for signal divergence. Second, female *S. bombifrons* choose heterospecific mates only in low-water conditions (Pfennig, 2007). In deep ponds, which dry slowly, the advantage of decreased development time is outweighed by the cost of reduction in fecundity. Thus, there are only a very limited set of circumstances where it is actually beneficial to hybridize and selection to avoid it may still be strong enough to cause signal divergence.

Despite the evidence for variation in selection for RCD as a driver of signal divergence, there are three alternative, though not mutually exclusive, explanations for the divergence in *S. bombifrons'* calls. These include: 1.) Genetic drift as *S. bombifrons* expanded into the desert ecoregion; 2) An environmentally-driven selective pressure not related to RCD; and 3) The possibility that the two call types are representative of cryptic species. Although each of these may have some role in signal divergence, we maintain that they are not the primary cause.

The range expansion of *S. bombifrons* south, from Oklahoma into the desert southwest, suggests that genetic drift is one possible explanation for the signal divergence found in the desert ecoregion. It is possible that through bottlenecks and mutation, the calls changed, and as a result, the toads that made it into this ecoregion had drastically different calls than those further

North. However, this seems an unlikely explanation as there is low variation in call parameters within slow-calling *S. bombifrons*, and there are no recorded calls in that region that approach those of fast-calling *S. bombifrons*. This means that unless previously existing variation has subsequently been lost, there would be little variation for selection to act on or for bottlenecks to isolate. Our inability to find a gradual transition from slow to fast calls is also a counter-indication of drift during range expansion.

A second possibility is that habitat is driving signal divergence in *S. bombifrons* through a process other than RCD. One of the most common explanations for signal divergence between environments is what is known as sensory drive (Endler, 1992), where differences in signal propagation between environments select for differences in signal design. However, the shortgrass prairie and desert are very similar in terms of signal propagation in that they are wide, open habitats, and are unlikely to select for differences in calls. This also does not explain why we see a concurrent shift in calling location. Shifting from calling from throughout the pond to calling from the edge of the pond is unlikely to affect signal propagation.

It is also possible that signal divergence is not due to direct selection, but rather that the different selective environments caused changes to some other aspect of male morphology that inadvertently altered in call type. However, here again, the potential change in morphology does not explain the additional change in calling location, unless all of these characteristics are linked—a possibility that seems unlikely.

It is also possible that RCD is driving signal divergence, but not the RCD that we have put forward. For example, it is possible that signal divergence is due not to reinforcement with *S. multiplicata*, but rather that it is an adaptation to reduce signal interference from the loud *Anaxyrus* species found calling in the fast call region. However, this possibility has not been

supported by lab studies. We performed a study testing female latency to locate male calls in noisy environments and failed to find any difference in the ease of call localization between the fast- and slow-call type (O'Brien, unpublished). Thus, the possibility of RCD due to signal interference is not well supported.

The last of the alternative possible explanations for signal divergence is the possibility that the two call types represent cryptic species, a possibility first raised by Pierce (1976). However, we were unable to find evidence of reproductive isolation in this system (see Appendix B), and previous genetic research has indicated that the two are the same species (Sattler, 1980). Still, the differences in call type and the lack of gene flow between the two regions, coupled with the existence of a fast call type of *S. intermontana* (O'Brien, unpublished data) suggest this possibility warrants further investigation.

Despite the alternative explanations, variation in the strength of RCD seems the most likely cause for call divergence in *S. bombifrons*. Previous research has shown that the strength of RCD can vary throughout a range of sympatry due to the risk, cost, and frequency of hybridization in a system. Here, using the spadefoot toad as a model system, we have shown that these factors need not act in isolation, but rather that they can vary in concert with one another, facilitated by changes in ecosystem and climate throughout the range sympatry.

It is unlikely that this is a rare occurrence. Regions of sympatry frequently cross multiple ecosystems, and variation in sexual signals throughout a species' range is surprisingly prevalent (e.g. Bernal et al., 2005; Gabor and Ryan, 2001). Future studies of RCD should consider the possibility that patterns of divergence may not be found throughout the entire range of sympatry, but rather in the regions of sympatry where the climate and ecosystem increase the strength of selection for RCD and facilitate signal divergence. Considering boundaries in ecoregion when

sampling will improve detection of RCD and improve our understanding of how variation in signaling can occur.

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CHAPTER 2: CALL STRUCTURE DETERMINES RESPONSE TO SIGNAL INTERFERENCE IN THE SPADEFOOT TOAD, GENUS *SPEA*

Introduction

Breeding aggregations, where males gather in large numbers and signal to attract mates, are found commonly throughout the order anura (Gerhardt and Huber, 2002). However, despite their ubiquity, these dense aggregations make for a very difficult signaling environment. Not only is there heightened competition for mates (Brumm, 2013), but males in breeding aggregations are also often faced with the problem of signal interference where the signals of competing males obscure one another (Brumm, 2013). This interference is highly detrimental to males, as females from multiple species have been found to strongly discriminate against overlapped calls (Schwartz and Wells 1983a, 1984; Wells and Schwartz 1984).

There are a number of adaptations that have been identified in chorus-breeding anurans to reduce signal interference and enhance signal attractiveness in the face of increased competition. These can be microevolutionary changes, such as alterations in the timing or location of signaling (Gerhardt and Huber, 2002) or facultative adjustments, where males temporarily alter their calls in response to the chorus environment (Klump and Gerhardt, 1992). Facultative adjustments may be in response to the noise level of the chorus as a whole, or, often, they are made in response only to the calls of nearest neighbors (Gerhart and Klump, 1988; Brush and Narins 1989; Schwartz 1993).

The most common response to the calls of neighboring anurans is adjustment of the fine-scale temporal properties of a call (Klump and Gerhardt, 1992) such as altering the call rate

(Wagner, 1989), creating more complex calls (Wells and Schwartz 1984), lengthening call duration (e.g., Wells and Taigen, 1986; Martínez-Rivera and Gerhardt, 2008), or increasing the space between calls or pulses (e.g. Martínez-Rivera and Gerhardt, 2008; Schwartz and Wells 1985). Males may also adjust the spectral components of their calls, though this is less common (e.g. Wagner 1989b; Bee and Perrill 1996; Bee and Bowling 2002; Howard and Young 1998). These facultative adjustments are significant in that they add a layer of complexity to sexual selection. This is particularly true if males adjust the call parameters used in female mate choice or species recognition (e.g. Botto, 2016).

However, despite the large body of work describing how males adjust their calls in response to interference, there has been little research into why there is such variation in which adjustments different species make (but see Grafe, 2003): Why do some species alter one call parameter while other species change different ones? We hypothesized that call structure likely plays an important role in determining the best strategy of signal adjustment for avoiding signal interference.

To explore the role of call structure in driving differences in facultative call adjustment we used the spadefoot toad (genus *Spea*) as a model system. We compare the calls of the New Mexican spadefoot toad (*Spea multiplicata*) and two alternate call types of the plains spadefoot toad (*Spea bombifrons*) which has both a "fast-call type" and a "slow-call type" (see Chapter 1). The calls of these three groups are structurally similar in that they consist of pure, tonal calls that are composed of repeated pulses. However, they exhibit slight variations in temporal structure and spectral frequency that make them clearly distinct. As a result, this system offers an excellent opportunity to explore how differences in call structure may lead to differences in facultative responses to interference.

For the purposes of this study, we focused on two primary drivers of call variation in noisy breeding aggregations: the distance from the focal male to the nearest calling male and the total number of calling *Spea* males in the pond. Together, these two parameters indicate the degree of interference that the focal male is experiencing. The "Total *Spea*" parameter provides an indication of the general background noise level that may interfere with signaling (Schneider et al. 1988; Schwartz 1993; Grafe 1996; Martínez-Rivera and Gerhardt, 2008) while the distance to the nearest neighbor parameter ("Nearest neighbor") gives a sense of more direct interference. Previous research has shown that spatial separation is a key component to successful sound-source segregation and close males are more difficult for females to distinguish between during call interference (Schwartz and Gerhardt, 1989). In agreement with this, research has shown that males typically attend to their nearest neighbor for call modification (Gerhart and Klump, 1988; Brush and Narins 1989; Schwartz 1993).

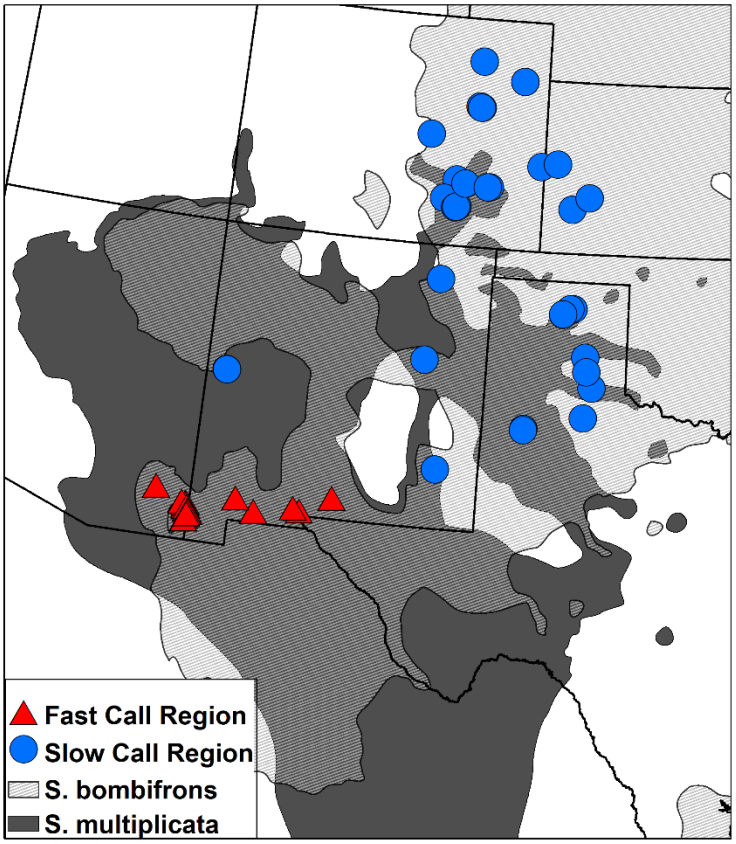

Figure 2.1 *S. bombifrons* and *S. multiplicata* have a large region of range overlap in the southwestern United States. Within its range, *S. bombifrons* has two distinct call types: A fast call type found in southern New Mexico and Arizona (blue triangles), and a slow call type found throughout the rest of its range (purple circles).

We first compare the effects of these two aspects of call environment (total calling *Spea* and distance to nearest neighbor) on male calls. We then discuss how differences in call

structure between the three groups (fast-call *S. bombifrons*, slow-call *S. bombifrons* and *S. multiplicata*) may contribute to differences in how males alter their calls in response to interference.

Methods

Study System

The plains spadefoot toad (*Spea bombifrons*) and New Mexican spadefoot toad (*Spea multiplicata*), are congeneric species that coexist in sympatry for a large portion of their range (Powell, 2016). Both species are explosive breeders that share breeding sites in the ephemeral ponds that form following heavy rains. The males call for at most a few days a year and, in some parts of their range, they call for only a single night (Bragg, 1965). Both *S. bombifrons* and *S. multiplicata* show a pattern of call divergence with significantly faster calls in the part of their range that stretches into the Chiricahua desert (Chapter 1; Figure 2.1). However, the difference in call type is much more extreme for *S. bombifrons*, and for this reason, we focused our comparisons on the slow call type of *S. multiplicata*, and both fast-call type and slow-call types of *S. bombifrons.*

Of the three species we studied, the calls of *S. multiplicata* are the slowest, with long call durations, long intercall intervals, and audible space between pulses (Figure 2.2). The slow call type of *S. bombifrons* is slightly faster than the call of *S. multiplicata* with a shorter duration, shorter intercall interval, and less separated pulses. The fast call type of *S. bombifrons* is the fastest of the three with very short calls, rapid pulse rates with unseparated pulses, and short intercall intervals (Chapter 1).

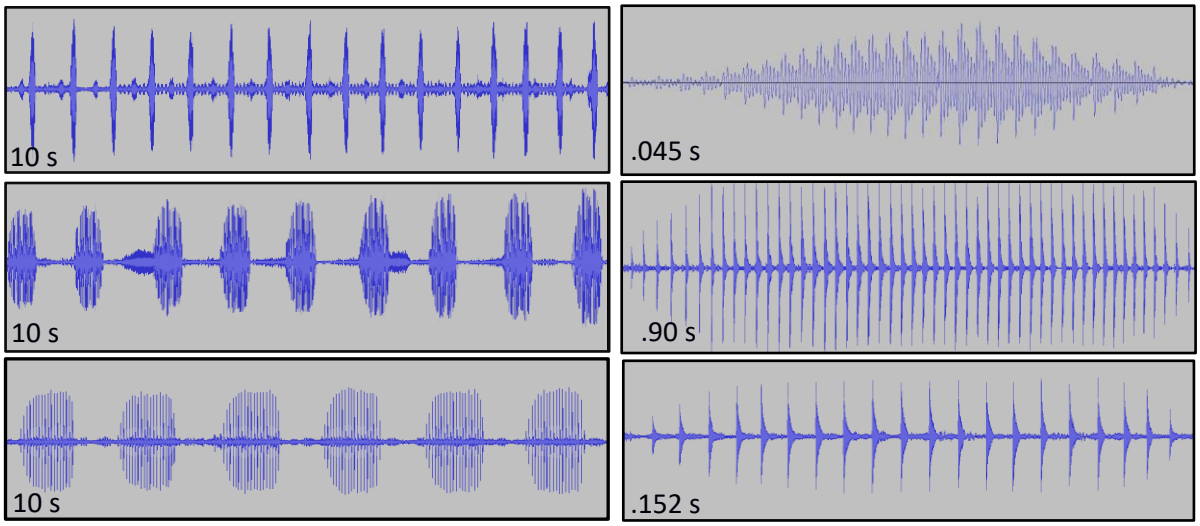

Figure 2.2 Waveforms of *S. bombifrons* fast call (top) *S. bombifrons* slow call (middle) and *S. multiplicata* (bottom). The panels on the left show a ten second call series, while the right show a single call

Field collections

Between 2016 and 2017 we recorded the calls of 350 spadefoot toads (40 fast-calling *S. bombifrons*, 210 slow-calling *S. bombifrons*, and 100 *S. multiplicata*) from 54 different populations. These ponds were located throughout Texas, Colorado, Kansas, New Mexico, and Arizona. At each pond, we estimated the total number of calling *Spea* and then recorded a selection of calling males for a minimum of 30 seconds at a sampling rate of 44.1 KHz. For a subset of the recorded males (85 slow-calling *S. bombifrons*, 24 fast-calling *S. bombifrons*, and 43 *S. multiplicata*), we also estimated the distance from the focal male to the nearest calling male. We caught, weighed, and measured each calling male that we were could (a total of 202 males), and collected a DNA sample in the form of a toe clip.

Call analysis

We analyzed each call to determine call rate (calls/minute), pulse rate (pulses/second), call duration (seconds), intercall interval (seconds) and call effort (time spent calling/minute)

using Audacity® sound analysis software 2.1.3 (Audacity team, 2018). Dominant frequency (Hz) was measured using Raven Pro 1.5 (Bioacoustic Research Program, 2014) software. We used a fast fourier transformation with a hamming window, a hop size of 82, and a DFT of size 2048.

Genotyping

S. bombifrons and *S. multiplicata* are known to hybridize, and hybridization can influence call characteristics (Blair, 1955; Pfennig, 2000; Lemmon, 2009; O'Brien, unpublished data.) In order to ensure that the presence of hybrids did not influence our results, we genotyped each toad we were able to catch (a total of 202 males), and then extrapolated our findings to identify hybrids among the non-genotyped males.

Using the protocol established in Pfennig et al. (2012), we determined species identity using a suite of 9 nuclear markers and one mitochondrial marker. We then used JMP Pro 13 (SAS Institute Inc., Cary, NC) to perform a PCA on a cross-correlation matrix including standardized values of call rate, pulse rate, dominant frequency, and pulse number. We used normal mixture clustering and compared AICc values to determine the most likely number of clusters in sound space. We then chose the model with the lowest AICc value by a minimum of 4 AICc units, or in the case of a tie, we chose the model with the fewest number of clusters. Based on our genotyped samples, these clusters closely reflected a combination of call type (fast or slow) and species (*S. bombifrons, S. multiplicata,* or hybrid.) Known hybrids and any ungenotyped individuals that clustered with hybrids in our PCA were eliminated from further analysis.

Statistics

We performed analysis of variance (employing the 'Anova' function in the 'car' package in R 3.4.1) on linear mixed models to measure the effects of distance to the nearest neighbor and total calling *Spea*, on male calls. The call parameters we tested included pulse rate, call rate, dominant frequency and call duration. For call rate, pulse rate, and call duration, we included water temperature as a covariate, as these parameters can be affected by temperature (Gerhardt and Huber, 2002). Similarly, dominant frequency is influenced by body size and shows a latitudinal cline (in addition to changes in body size) so we included these covariates in our model of dominant frequency (Zweifel, 1968; McClelland et al., 2006). All models included the pond from which the male was collected as a random effect.

Results

All three groups of *Spea* showed variation in their call parameters in response to the distance to the nearest neighbor and the total calling males. However, which call parameters

Table 2.1 Aspects of chorus environment with significant effects on call parameters. We have indicated whether a coefficient was positive or negative.

Call Parameter	Fast *S. bombifrons*	Slow *S. bombifrons*	*S. multiplicata*
Call Rate	Nearest Neighbor (+)	N/A	Total *Spea* (-) †
Pulse Rate	N/A	Total *Spea* (-)	Total *Spea* (-) †
Dominant Frequency	N/A	N/A	N/A
Duration	N/A	N/A	Nearest Neighbor (-) Total *Spea* (+) †2
Intercall Interval	Nearest Neighbor (-)	N/A	Nearest Neighbor (-) Total *Spea* (+) †2
Call Effort	Nearest Neighbor (+)	Nearest Neighbor (-)	N/A

† Total *Spea* was significant with the inclusion of an outlier. Significance was lost with the omission of that outlier.
†2 Nearest neighbor was significant with and without the outlier.

were affected varied between the three call types (Table 2.1).

In slow-calling *S. bombifrons*, we found a nearly, but not quite significant effect of distance to the nearest neighbor on call duration, with decreasing distance leading to increasing duration (χ^2= 3.284, df=1, p=.057). This increasing call duration was likely the cause of higher call effort in males calling closer together (χ^2=4.912, df= 1, p<0.05, Figure 2.3). Pulse rate also changed in response to chorus environment, but this call parameter changed in response to the total number of males rather than the nearest neighbor. As the total number of calling males increased, the pulse rates decreased, and this held both with (χ^2=6.211, df=1, p<0.05; Figure 2.4), and without (χ^2= 6.982, df=1, p=0.008) the inclusion of an outlier pond that had an unusually high

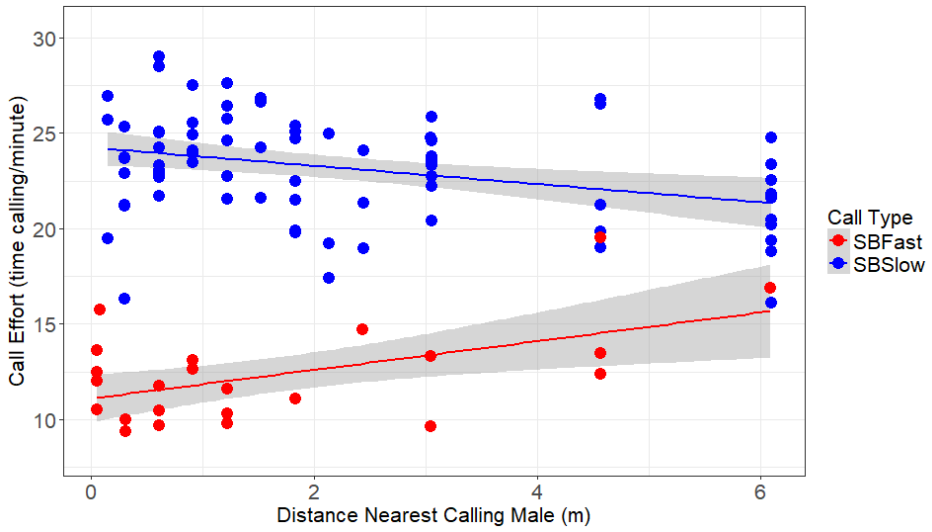

Figure 2.3 Fast and slow calling *S. bombifrons* showed opposite patterns of call effort with increasing distance from the nearest neighboring male. Values are not temperature corrected

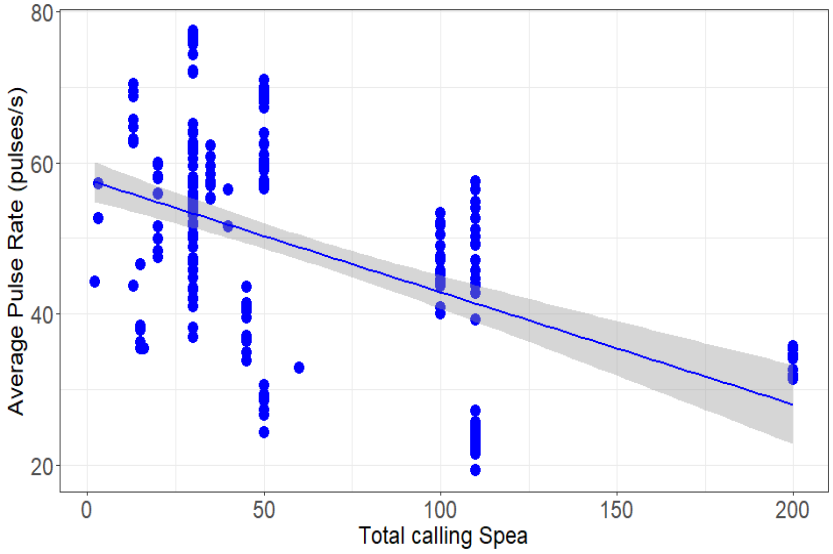

Figure 2.4 In slow calling *S. bombifrons* increasing total males led to a decrease in pulse rate. This pattern held both with and without the inclusion of the pond with 200 individuals. Values are not temperature corrected.

number of total males. We found no effect of total males or distance to the nearest neighbor on call rate, intercall interval, or dominant frequency in this group.

Despite being the same species, fast-calling *S. bombifrons* showed a completely different set of call modifications in response to interference than slow-calling *S. bombifrons*. Fast-calling *S. bombifrons* did not show changes in pulse rate or duration ($p>0.05$), but instead showed a change in call rate. Males calling closer together had longer intercall intervals ($\chi^2= 9.7603$, df=1, $p=0.002$) and, as a result, slower call rates ($\chi^2= 22.568$, df=1, $p<.001$). This meant that fast-calling *S. bombifrons* showed changes in call effort that were the opposite of those seen in slow-calling *S. bombifrons*, with increasing call effort in response to decreasing interference ($\chi^2= 8.980$, df=1, $p=0.003$; Figure 2.3). Dominant frequency did not change in response to any of the aspects of chorus environment we tested.

In *S. multiplicata*, males altered their pulse rates in a manner similar to slow-calling *S. bombifrons*, with increasing total males leading to decreasing pulse rate ($\chi^2=4.790$, df=1, $p<.05$).

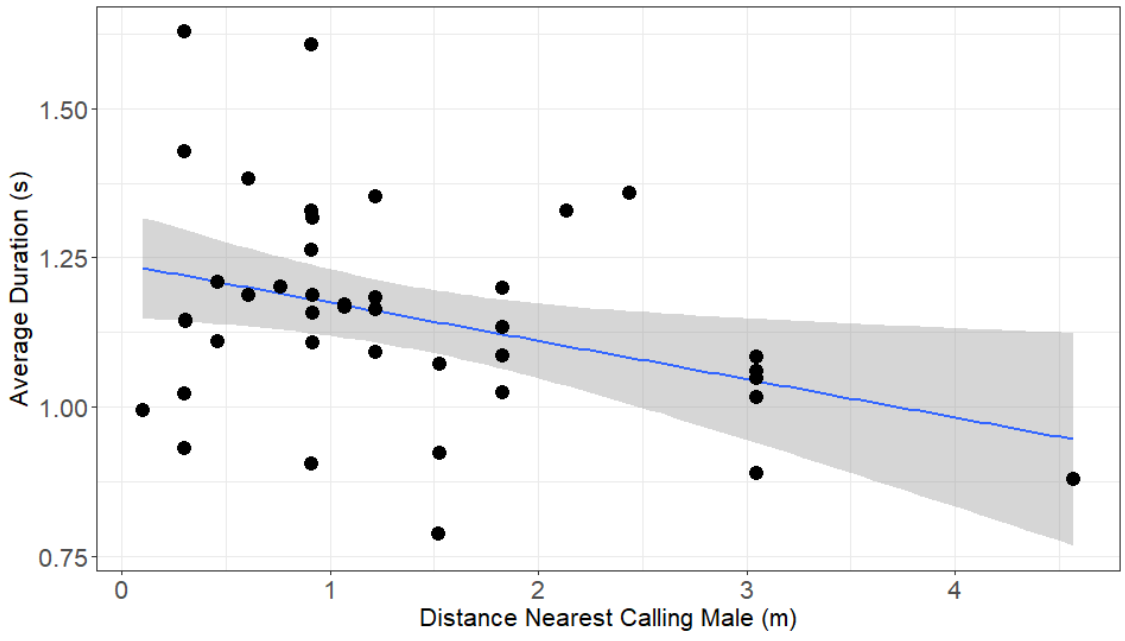

Figure 2.5 *S. multiplicata* showed decreasing call duration with increasing distance from the nearest calling male

Although this significance was lost with the exclusion of an outlier, the effect remained nearly significant (χ^2=3.293, df=1, p=0.070). The nearest calling male also had a nearly, but not quite significant effect on pulse rate (χ^2=3.383, df=1, p=0.066). There was a significant effect of total males on call rate in *S. multiplicata*, with this species decreasing their call rate in response to increasing numbers of males (χ^2=4.3112, df=1, p<0.05). Both duration and intercall interval were affected by call rate and nearest neighbor in *S. multiplicata*, with increasing interference leading to increasing duration (*Total males*: χ^2=7.821, df=1, p=0.005; *nearest neighbor* χ^2=9.043, df=1, p=0.003) and increasing intercall intervals (T*otal males*: χ^2=4.747, df=1, p=0.029; *Nearest neighbor*: χ^2=6.6703, df=1, p=0.010). However, the effect of total males was lost with the exclusion of an outlier pond with an unusually large number of total males (*Duration:* χ^2=0.565, df=1, p=0.414; *Intercall interval:* χ^2=0.6107, df=1, p=0.434). We found a marginally significant effect of proximity to the nearest neighbor on dominant frequency (χ^2=3.691, df=1, p=0.055). In contrast to *S. bombifrons, S. multiplicata's* call effort was not affected by signal interference.

Discussion

Like many species of anurans, members of the genus *Spea* alter their call parameters in response to signal interference. However, the ways in which members of this genus alter their calls differ across species and call type. These differences are not connected to phylogenetic relatedness, but rather appeared to be primarily the result of differences in call structure.

Slow-calling *S. bombifrons*, and *S. multiplicata* have long calls with clearly discrete pulses, characteristics which make them structurally more similar to one another than they are to

fast-calling *S. bombifrons*. Relatedly, these two groups showed a unique response to signal interference not found in fast-calling *S. bombifrons* where they reduced their pulse rates in response to increasing total numbers of calling *Spea*. Although the effect was not quite significant in slow-calling *S. bombifrons*, both species also showed an increase in duration in response to increasing interference, a modification which was not found in fast-calling *S. bombifrons*.

The changes to calling that we observed in response to signal interference in slow-calling *S. bombifrons* and *S. multiplicata* are consistent with the findings of previous interference research. Altering pulse rate and call duration are common responses to interference in anurans (e.g. Martínez-Rivera and Gerhardt 2008 Wagner, 1989; Wells and Taigen, 1986; Martínez-Rivera and Gerhardt, 2008), and are thought to improve mating success. Increasing duration is thought to either improving female ability to localize the call (the call detection hypothesis) or to increase the attractiveness of the call by improving the chance that at least part of it is unobscured by interfering signals (the interference risk hypothesis; Schwartz et al. 2001; Schwartz et al., 2013). The decrease in pulse rate is likely evidence of pulse interdigitation. In many species, when males are faced with call overlap, they will alternate pulses to avoid having their pulses obscured (e.g. Martínez-Rivera and Gerhardt, 2008; Grafe, 2003).

In fast-calling *S. bombifrons,* we found that males altered their calls in ways that differed from the other two groups. Perhaps most surprising was the change in call rate that we observed. In many species of anurans, females select males on the basis of call rate, with faster call rates indicating males in better condition (Gerhardt and Huber, 2002). One might expect that males calling close together would increase their call rate in order to more effectively compete with their neighbors. However, in fast-calling *S. bombifrons,* we found the opposite to be true. This

suggests female discrimination against overlapped calls is strong enough to encourage slower call rates despite other forces selecting against it. Alternatively, it is also possible that the decrease in call rate is compensated for later on. Males may attract females from a distance with unobscured calls, but then ramp up their call rates without regard to call overlap to demonstrate fitness when the female approaches. We often observed such behavior in the field (O'Brien, personal observation).

The differences in response to interference between *Spea* with slower calls (*S. multiplicata* and slow-calling *S. bombifrons)* and *Spea* with faster calls (fast-calling *S. bombifrons*) are likely due to differences in call structure. The calls of fast-calling *S. bombifrons* are much shorter than those of either of the other two groups and have almost no space between pulses. This lack of space means that males would have to reduce their pulse rates by a terrific amount to successfully avoid pulse overlap—a change that may be physiologically difficult and may render their calls unrecognizable and/or unattractive to females. Instead, it may be more advantageous for this species to reduce call overlap entirely by increasing the space between calls.

This hypothesis is consistent with field observations. We often observed multiple fast-calling *S. bombifrons* alternating calls with one another without overlap (Figure 2.6). In contrast, when we observed any more than two slow-calling *S. bombifrons* or *S. multiplicata* calling in close proximity, the males were unable to avoid overlap and instead during any overlap they would interdigitate pulses (Figure 2.6). For species that have sufficiently discrete pulses and long calls, altering pulses may be a more effective strategy than adjusting call rates to avoid any call overlap.

Our finding that only males with slower calls increase their duration in response to interference lends support to the interference risk hypothesis, which states that increased duration serves to improve the chance that at least some of the call is unobscured. If this modification served to improve call localization, as the call detection hypothesis suggests, we would expect to see an increase in duration in all three groups, rather than the alternate strategies between groups with different call structures that we actually observe.

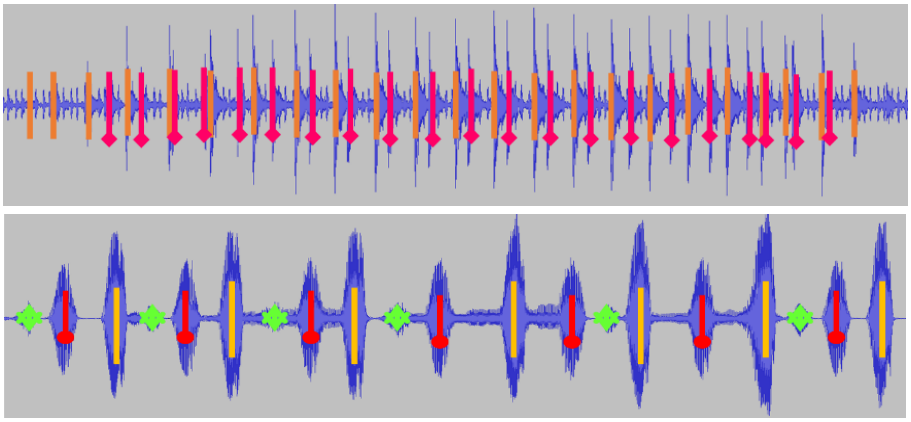

Figure 2.6 Alternate strategies to avoid call overlap between different call structures. The top panel shows a field recording of two *S. multiplicata* calling simultaneously. It shows a single call with interdigitated pulses. The bottom panel shows three fast-call *S. bombifrons* all alternating calls. It shows a series of alternating calls. Different individuals have been marked with colored bars.

As a result of the differences in how the various *Spea* groups modify call duration and call rate, there were also differences in how call effort (total time spent calling per minute) varied in response to interference. We found that while slow-calling *S. bombifrons* have higher call efforts when calling close to other males, fast-calling *S. bombifrons* had the reverse effect. *S. multiplicata*, which increase not only their call duration but also their intercall interval, showed no change in call effort in response to their nearest neighbors. This may explain why both of these characteristics are modified: increasing call effort is energetically expensive, and the simultaneous changes to duration and intercall interval reduces the cost of increasing call duration.

The differences in call effort across these three groups are noteworthy, as they suggest differences in the energetic cost of interference across the three groups. Specifically, our results suggest that slow-calling *S. bombifrons* males pay the greatest cost for calling in dense aggregations. Alternatively, it is also possible that the energy saved by reducing call effort in fast-calling *S. bombifrons* was put towards other means of increasing signal clarity such as producing louder calls. This possibility merits further attention, as the use of amplitude modulation in response to signal interference is one that has seen little attention in this area of research (Brumm, 2013).

The differences in response to interference between these three groups suggests that the structure of sexual signals may play a profound role in how animals respond to problems of signal interference. However, despite the strong results of this study, it should be noted that our findings were entirely correlational. Future studies should confirm that these differences in call characteristics are indeed causational, as well as further investigating the metabolic costs and benefits of different signal structures and strategies for reducing signal interference.

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CONCLUSION

Explaining the origins of diversity is an enduring goal in evolutionary ecology, and in few places is this diversity as striking as in sexual signals. In this thesis, using spadefoot toads as a model system, I have explored two aspects of signal diversity, focusing on how interactions between both members of the same and of different species can contribute to signal diversity.

First, I explored interactions between species by examining changes in sexual signals due to reproductive character displacement (RCD). In particular, I focused on how differences in ecosystem and climate can promote variation in RCD throughout a range of sympatry. Second, I looked at interactions both between and within species by examining the importance of signal design in determining how males facultative alter their calls in response to interference from competing males.

My results showed that differences in ecoregion have the potential to affect many of the factors that influence the strength of RCD and may play a key role in determining the degree of signal divergence throughout a range of sympatry. I also found that both the nearest calling male and the total chorus size can influence signaling, and that the structure of the signal influences how males adjust their calls to reduce signal interference.

Future work should continue to explore the potential for ecoregion to drive variation in RCD throughout the range of sympatry. This may entail revisiting previously observed variation in divergence throughout a range of sympatry, or actively seeking out ranges of sympatry that cross multiple ecoregions. Furthermore, this should also guide future research by encouraging researchers to consider boundaries in ecoregion when deciding where to sample for RCD.

In terms of facultative adjustments to signal design, future studies should build on our results by following a similar model of comparing the calls of closely related species that vary subtly in structure. For example, future research could focus on differences in call complexity or dominant frequency between closely related calls. This will provide further insight into why there is variation in how animals respond to signal interference. Additionally, future research should focus on the broader metabolic impacts of signal adjustment and the implications this may have for how males space themselves in a chorus. (E.g. Does proximity to nearest neighbor reflect condition?)

In the spadefoot system specifically, future research in facultative signal adjustments should focus on manipulative experiments that confirm the cause and effect relationship of signal variation. It should also further explore the potential for amplitude modulation as a response to signal interference in fast-calling *S. bombifrons*. Additionally, it would be interesting to know the extent to which females select males on the basis of their ability to rapidly increase call rate as the female approaches.

Future work in microevolutionary change in *Spea* should expand beyond *S. bombifrons* and *S. multiplicata* and address how the call variation in *S. bombifrons* fits into the broader context of the phylogeny. In particular, further exploration of signal design in the closely related *Spea intermontana* which exhibits a fast-call type in Wyoming (Wyoming Fish and Wildlife Department, personal communication), has the potential to provide great insight into signal divergence in this species.

APPENDIX A: TABLES AND FIGURES

	PC 1	PC 2	PC 3	PC 4
Pules number	0.576	0.783	-0.232	0.0413
Pulse Rate	0.947	-0.183	-0.176	-0.196
Call Rate	0.871	-0.411	-0.207	0.170
Dominant frequency	0.780	0.103	0.617	0.017
Eigenvalue	2.597	0.826	0.508	0.069
Percent Variation	64.921	20.640	12.707	1.732
Cumulative Percent	64.921	85.561	98.268	100.000

Table A1 PCA results for *S. bombifrons* and *S. multiplicata* call parameters.

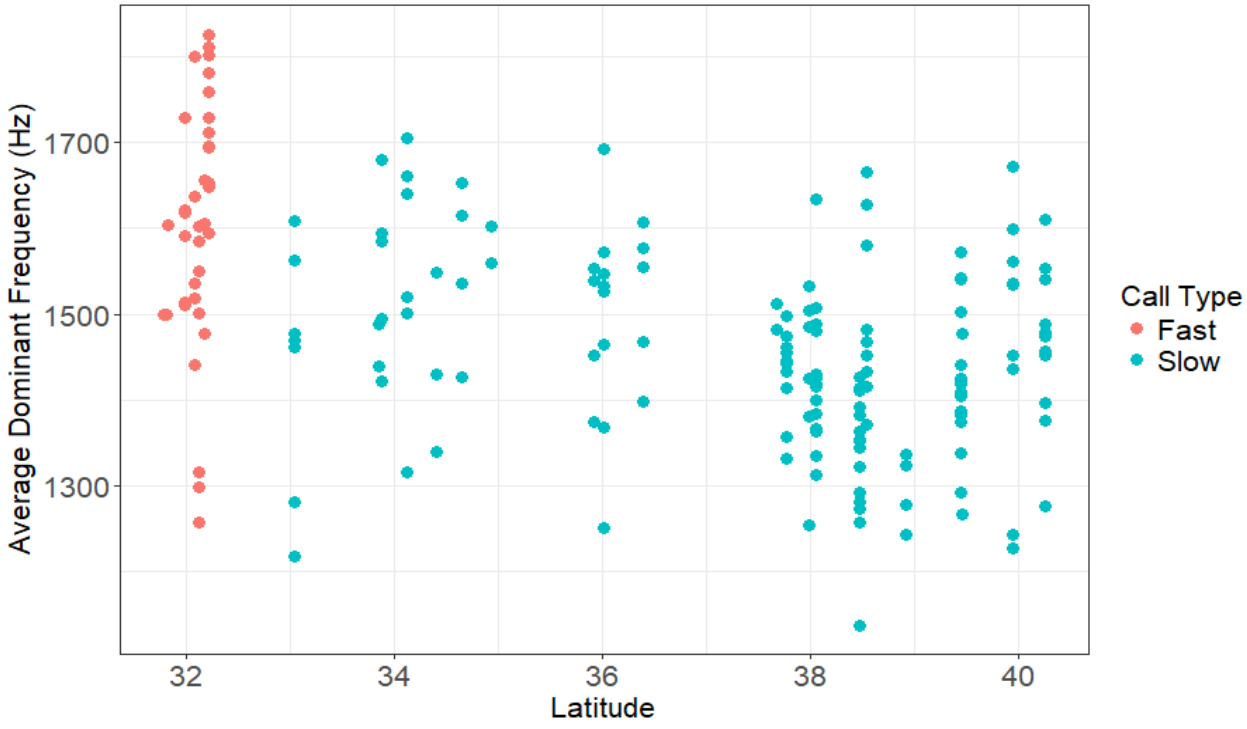

Figure A1 Average dominant frequency shows evidence of a latitudinal cline in *S. bombifrons*

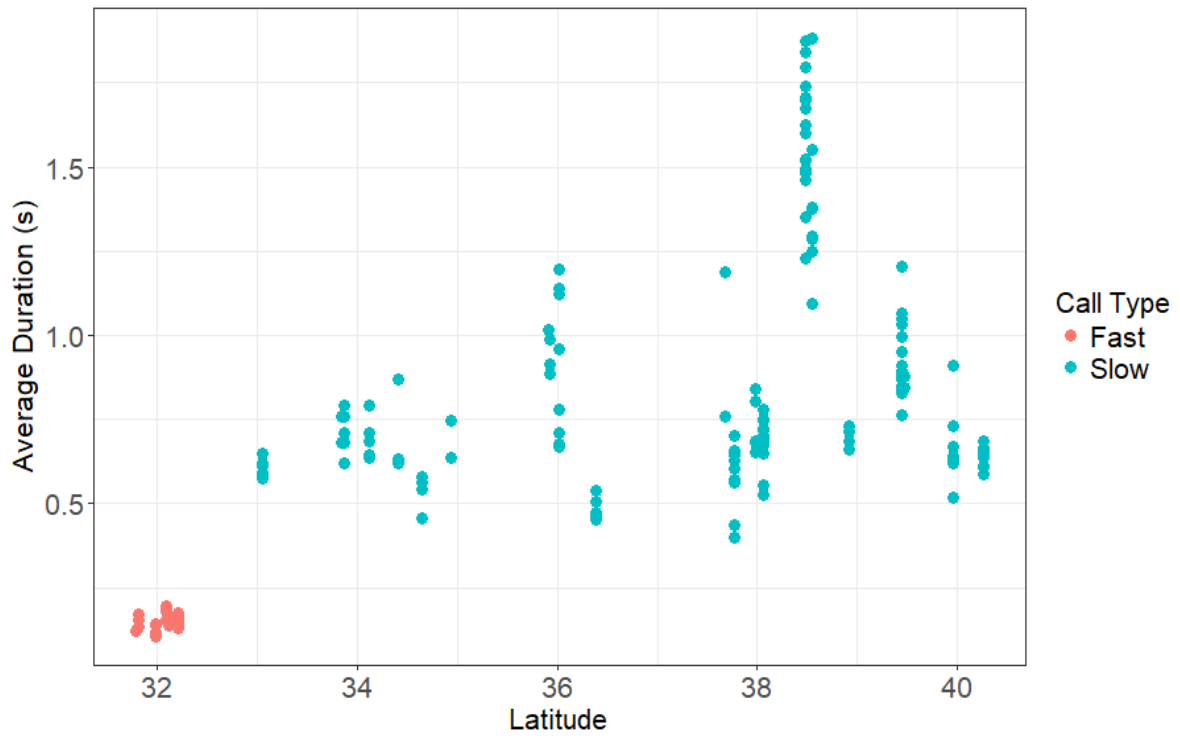

Figure A2 Average duration did not show evidence of a latitudinal cline in *S. bombifrons*

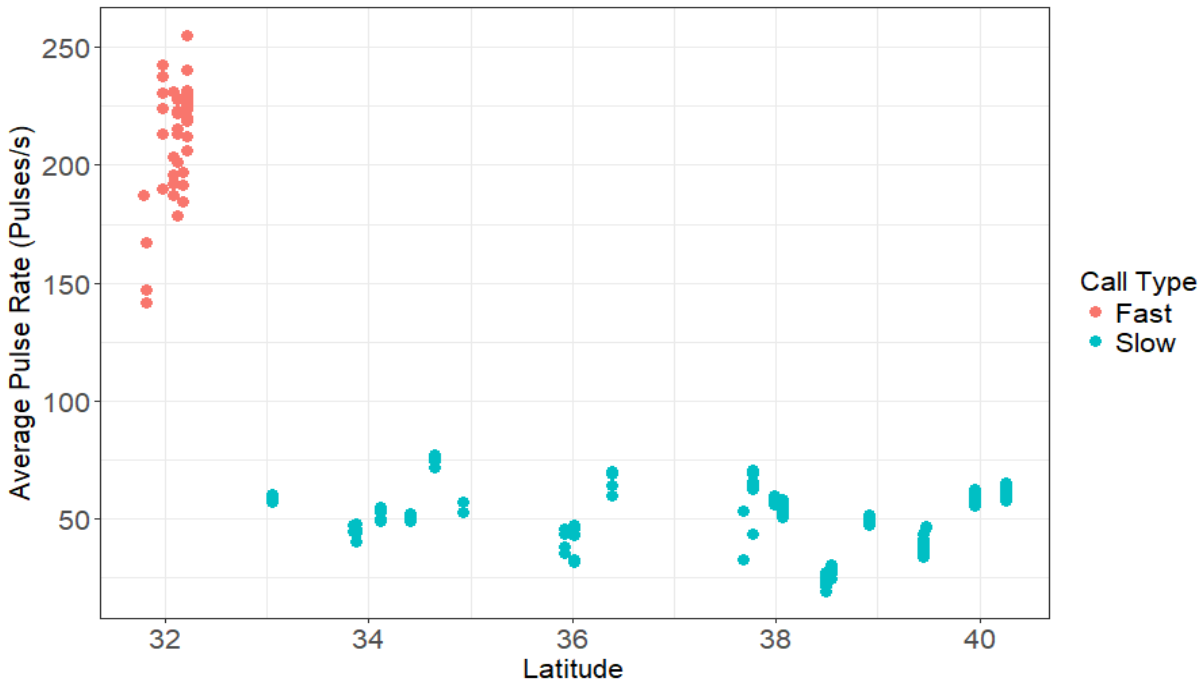

Figure A3 Average pulse rate did not show evidence of a latitudinal cline in *S. bombifrons*

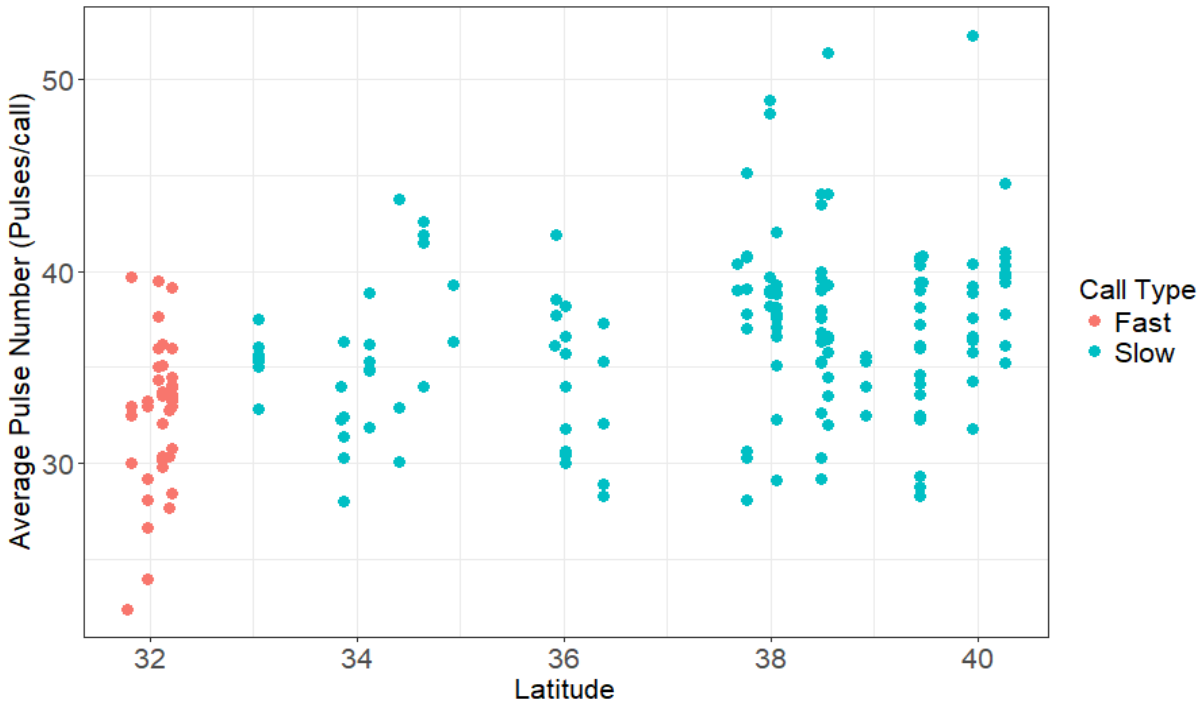

Figure A4 Average pulse number showed evidence of a latitudinal cline in *S. bombifrons*

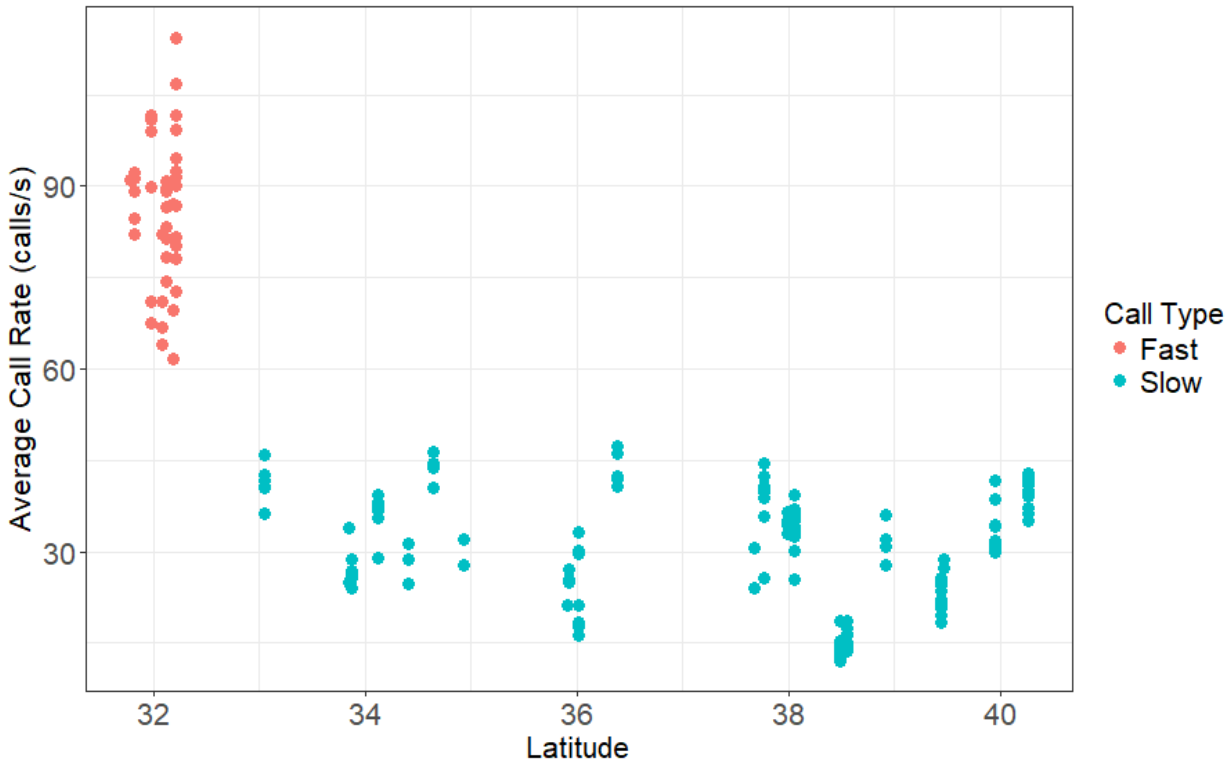

Figure A5 Average call rate did not show evidence of a latitudinal cline in *S. bombifrons*

APPENDIX B: *PRELIMINARY DATA:* REPRODUCTIVE ISOLATION DUE TO REPRODUCTIVE CHARACTER DISPLACEMENT WITHIN A RANGE OF SYMPATRY

Introduction

Our results in Chapter 1 suggest that reproductive character displacement (RCD) may have a more complicated role in speciation than previously thought. RCD has long been thought to cause speciation due to reduced gene flow between displaced and non-displaced regions (Howard 1993; e.g. Hoskin et al. 2005). However, our results suggest that there may also be differences in RCD within a region of sympatry, meaning that reproductive isolation could develop between sympatric populations. To investigate this, we tested for pre-zygotic isolation due to female choice between the fast-call type and slow-call type of *S. bombifrons.*

Methods

Stimuli creation

Between 1996 and 2017 we recorded the calls of 191 *S. bombifrons* males from the fast- and slow-call regions of their range. These ponds were located throughout Texas, Colorado, Kansas, New Mexico, and Arizona. We analyzed each call to determine pulse number (pulses/call), intercall interval (seconds), and call duration (seconds) using Audacity® sound analysis software 2.1.3 (Audacity team, 2018). We measured dominant frequency (Hz) using Raven Pro 1.5 (Bioacoustic Research Program, 2014) software. For this analysis, we used a fast fourier transformation with a hamming window, a hop size of 82, and a DFT of size 2048. After our call analysis, we calculated the median values of call duration, intercall interval, pulse number and dominant frequency for the fast- and slow-call types of *S. bombifrons*.

Based on our field data, we then used Audacity® sound analysis software to generate calls with median call characteristics of the fast and slow call type. From our field recordings, we selected a call with a dominant frequency as close as possible to the median value and clipped a single pulse from that call. We then combined pulses to match the median pulse number and call duration of each call type, and the entire call was structured to fade in for the first half of the call and fade out for the second half. Calls were separated by a period of silence matching the median inter-call interval.

Experiment

We tested 30 *S. bombifrons* females (15 from the fast call region and 15 from the slow call region) collected from Colorado, Nebraska, Oklahoma, Texas, and Arizona between the years of 2011 and 2017 and returned to the University of North Carolina, Chapel Hill. The toads were housed in sand-filled containers in a temperature stabilized-room and fed *ad libitum*. *Spea* are nocturnal, so the toads were kept on a reverse light dark cycle to ensure they were active during the time of testing. To ensure that the females were selective, we used only gravid females in our experiment.

We tested for preferences using standard phonotaxis protocol (e.g. Gerhardt, 1991; Wagner and Sullivan, 1995). Each trial took place in a circular cattle tank filled with approximately six inches of water and placed inside a sound-reducing chamber. The tank had speakers arranged 180 degrees from one another and approximately 1.5 m apart. At the center of the tank, directly between the two speakers was a small platform. To begin the trial, an observer placed the focal female in an opaque holding cell on the central platform and began the stimuli for a five-minute acclimation period. The stimuli consisted of a slow-call stimulus, played from

one speaker and a fast-call stimulus played from the other. The two stimuli were played in alternating 30-second intervals. We randomized both which speaker played which stimuli and which stimuli was played first between trials.

Using an infrared video camera, an observer watched the tank remotely, and after five minutes the focal female was released from the holding cell and allowed to move freely around the cattle tank. Female *Spea* choose their mates by touching them, so the observer watched the female until she touched a speaker. The touched speaker was counted as a choice, and we stopped the stimuli and ended the trial. If the female did not touch either Speaker after a period of 30 minutes, the trial was counted as a no-choice and the female was not re-tested. We analyzed our results both between and within call types using a chi-squared test.

Results

We found that there was no significant difference in choice between the fast- and slow-call types (X^2=1.9827, df=1, p>.05). However, we did find a nearly significant preference looking within each call type. Female *S. bombifrons* from the slow-call region showed a nearly significant preference for their local call type (X^2=.28571, df=1, p=.052) while fast-call *S. bombifrons* showed no preference (X^2=3.7692, df=1, p>.05).

Discussion

Although our results do not confirm that there is reproductive isolation between the two groups, they do suggest that there is a pattern of diverged preference between the two call types. We encourage future researchers to continue to explore this possibility.

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